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(54) Title: EF-Tu mRNA AS A MARKER FOR VIABILITY OF BACTERIA**(57) Abstract**

The present invention is related to the detection of bacteria, such as Mycobacteria, in human or animal body fluids such as blood, sputum and urine. The present invention provides a method for assessing the viability of bacteria such as *Mycobacterium tuberculosis* without the need for propagation of the bacteria. The method of the present invention is in particular useful for assessing the viability of Mycobacteria species such as are *M. tuberculosis* or *M. leprae*. With the present invention oligonucleotides are provided that can be used as primers and probes for the amplification of bacterial EF-Tu mRNA. The use of the oligonucleotides according to the invention is not limited to any particular amplification technique or any particular modification thereof. It is evident that the oligonucleotides according to the invention find their use in many different nucleic acid amplification techniques and various methods for detecting the presence of (amplified) bacterial EF-Tu mRNA.

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EF-Tu mRNA as a marker for viability of bacteria.

The present invention is related to the detection of bacteria, such as Mycobacteria, in human or animal body fluids such as blood, sputum and urine. The present invention provides
5 a method for assessing the viability of bacteria such as *Mycobacterium tuberculosis* without the need for propagation of the bacteria.

For example, tuberculosis (TB) caused by *Mycobacterium tuberculosis* is a major public health problem in many countries world-wide with particular significance in developing countries. Tuberculosis control programmes are faced with an increased burden of cases, a
10 shift towards diagnostically more difficult categories of patients such as extrapulmonary and smear-negative cases, and the emergence of multidrug-resistant strains of *M. tuberculosis*. Improved diagnosis would be a valuable contribution in the struggle to solve this global public health emergency.

The method of the present invention is concerned with the amplification of specific
15 nucleic acid sequences.

Nucleic acid amplification reactions promise to reduce the time for diagnosis from weeks to hours, while surpassing the sensitivity and specificity of the classical methods. Besides their potential value in diagnosis, amplification reactions offer the possibility of a rapid identification and drug-susceptibility determination. Amplification of DNA target molecules to a detectable
20 level by the polymerase chain reaction (PCR) is the best analyzed system for detecting *Mycobacteria*.

The "Polymerase Chain Reaction" (PCR) is described in European patent applications EP 200362 and EP 201148. PCR is a cyclic process which has double stranded DNA as target. Each cycle in the PCR process starts with the separation of a double stranded DNA
25 target in its two complementary strands. To each strand a primer will anneal and DNA polymerases present will extend the primers along the DNA strand to which it annealed thus forming two new DNA duplexes. When the reaction mixture is heated the strands of the DNA duplexes will be separated again and a new PCR cycle can start. Thus, the PCR process produces multiple DNA copies of a DNA target. Amplification using PCR, can also be based on
30 an RNA template. The actual PCR needs to be preceded by a reverse transcription step to copy the RNA into DNA (RT-PCR). However, if RT-PCR is used for the detection of transcripts differentiation of mRNA- and DNA-derived PCR products is necessary. DNase treatment prior to RT-PCR can be employed (Bitsch, A. et al., J Infect. Dis 167, 740-743., 1993; Meyer, T. et

al., Mol. Cell Probes. 8, 261-271., 1994), but sometimes fails to remove contaminating DNA sufficiently [Bitsch, A. et al., 1993].

More recently a different class of nucleic acid amplification methods namely the "transcription based amplification techniques" was developed. The techniques involve the transcription of multiple RNA copies from a template comprising a promoter recognized by an RNA polymerase. Said copies are used as input for further amplification. Such methods have been described by Gingeras et al. in WO88/10315 and Burg et al. in WO89/1050. Isothermal transcription based amplification techniques have been described by Davey et al. in EP 323822 (relating to the NASBA method), by Gingeras et al. in EP 373960 and by Kacian et al. in EP 408295 (the TMA method). Transcription based amplification reactions may also be performed with thermostable enzymes. Such a thermostable method is described in EP 682121 filed in the name of Toyo Boseki KK.

The isothermal transcription based nucleic acid amplification techniques have been utilized to detect mycobacteria, such as the NASBA method [Vliet, G.M.E. van der, Schukkink, R.A.F., Gemen, B. van, Schepers, P. and Klatser, P.R. (1993) Nucleic acid sequence-based amplification (NASBA) for the identification of mycobacteria. J. Gen. Microbiol. 139, 2423-2429.] and another transcription-mediated RNA amplification test (TMA)[Jonas, V., Alden, M.J., Curry, J.I., Kamisango, K., Knott, C.A., Lankford, R., Wolfe, J. and Moore, D.F. (1993) Detection and identification of Mycobacterium tuberculosis directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. 31, 241] both targeted at 16S ribosomal RNA.

Amplification reactions targeted at the 16S rRNA or the gene encoding it are usually directed to a conserved region which comprises species-specific variable sequences [Vliet, G.M.E. van der, Schukkink, R.A.F., Gemen, B. van, Schepers, P. and Klatser, P.R. (1993) Nucleic acid sequence-based amplification (NASBA) for the identification of mycobacteria. J. Gen. Microbiol. 139, 2423-2429., Jonas, V., Alden, M.J., Curry, J.I., Kamisango, K., Knott, C.A., Lankford, R., Wolfe, J. and Moore, D.F. (1993) Detection and identification of Mycobacterium tuberculosis directly from sputum sediments by amplification of rRNA. J. Clin. Microbiol. 31, 241]. They have the advantage that a single amplification reaction can identify the mycobacterial species. An additional advantage of the transcription-mediated RNA amplification assays targeted at 16S rRNA, is the high number of target molecules per cell - ± 2000; sensitivity is thereby favoured.

Since RNA, especially mRNA, has a generally much shorter half-life time than DNA, its detection may be useful for the assessment of the viability of mycobacteria [Moore, D.F., Curry, J.I., Knott, C.A. and Jonas, V. (1996) Amplification of rRNA for assessment of treatment response of pulmonary tuberculosis patients during antimicrobial therapy. *J. Clin. Microbiol.* 34, 5 1745-1749., Vliet, G.M.E. van der, Schepers, P., Schukkink, R.A.F., Gemen, B. van and Klatser, P.R. (1994) Assessment of mycobacterial viability by RNA amplification. *Antimicrob. Agents Chemother.* 38, 1959-1965.], which is relevant to the problems of resistance against drugs and contagiousness of the patient.

The present invention is based on the detection of mRNA encoding the elongation factor 10 EF-Tu.

The elongation factor EF-Tu is essential in (myco)bacterial translation. Elongation factors play an ancillary role in the elongation step of translation and are thus an indicator of the cell's metabolic activity. For every translation EF-Tu is required. The amount of EF-Tu protein can be as high as 50% of their total protein content in active proliferating cells.

15 EF-Tu encoding gene sequences (DNA) have been used as a marker to detect the presence of bacterial cells.

In EP133288 a method is disclosed for the detection of bacterial DNA with a probe comprising a base sequence of at least a portion of one of the strands of a tuf or fus gene. Southern blot hybridization of the digested mycoplasmal DNAs with the elongation factor (EF-20 Tu) gene tuf of *E.coli* was used as a basis to detect polymorphism in mycoplasma strains.[Yogev et al. *FEMS Microbiol.Lett.*, 50(2-3), 145-9, 1988].

A PCR based assays for the detection of *Mycoplasma tuberculosis*, and *Mycoplasma fermentans* using the gene encoding elongation factor Tu (tuf) as the target sequence had also been described [Berg et al.,*Mol.Cell.Probes*, 10(1), 7-14,1996 and Luneberg et al., 25 *J.Clin.Microbiol.*, 31(5), 1088-94, 1993].

The present invention, however, is concerned with the detection of EF-Tu mRNA as a marker for bacterial viability.

The present invention thus provides a method for the assessment of bacterial viability whereby mRNA coding for the elongation factor EF-Tu is used as a target in a nucleic acid 30 amplification reaction and the presence and/or amount of said mRNA is determined.

The presumably short-lived mRNA coding for the EF-Tu is most likely highly abundant in the (myco)bacterial cell and a decrease therein will indicate a decline in metabolic activity. Furthermore, because of the EF-Tu's essential role, it is plausible to assume that it is present in

all mycobacterial species, allowing the development of a general amplification system with species-specific primers and/or probes, analogous to the 16S rRNA NASBA design [Vliet, G.M.E. van der Schukkink, R.A.F., Gemen, B. van, Schepers, P. and Klatser, P.R. (1993) Nucleic acid sequence-based amplification (NASBA) for the identification of mycobacteria. J. Gen. Microbiol. 139, 2423-2429].

With the method of the present invention, preferably a transcription based amplification technique, such a NASBA, is used for the amplification of the bacterial EF-Tu mRNA. In contrast to RT-PCR, NASBA, which is based on RNA transcription by T7 RNA polymerase (Kievits et al., 1991; Compton, 1991), does not need differentiation between RNA- and DNA-derived amplification products since it uses RNA as its principal target.

The amplified products may be detected using a complementary labeled probe.

Numerous protocols have been described for the detection of amplified products [Klatser, PR. (1995) Amplification reactions in mycobacteriology. J. Microbiol. Meth. 23, 75-87.]. Preferably homogeneous assays are used, because they would allow amplification reaction mixtures to be sealed before amplification is initiated. One such system, electro-chemiluminescence (ECL), has already been successfully applied to detect amplified products in NASBA [Gemen, B. van, Beuningen, R. van, Nabbe, A., Strijp, D. van, Jurriaans, S., Lens, P., Kievits, T. (1994) A one-tube quantitative HIV-I RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labelled probes. J. Virol. Methods, 49, 157-167.].

The method of the present invention is in particular useful for assessing the viability of Mycobacteria species such as are M. tuberculosis or M.leprae.

With the present invention oligonucleotides are also provided that can be used as primers and probes for the amplification of bacterial EF-Tu mRNA.

The use of the oligonucleotides according to the invention is not limited to any particular amplification technique or any particular modification thereof. It is evident that the oligonucleotides according to the invention find their use in many different nucleic acid amplification techniques and various methods for detecting the presence of (amplified) bacterial EF-Tu mRNA. The oligonucleotides of the present invention can likewise be used in quantitative amplification methods. An example of such quantitative method is described in EP 525882.

The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides. Such oligonucleotides may be used as primers and probes.

Of course, based on the sequences of the oligonucleotides of the present invention, 5 analogues of oligonucleotides can also be prepared. Such analogues may constitute alternative structures such as "PNA" (molecules with a peptide-like backbone instead of the phosphate sugar backbone of normal nucleic acid) or the like. It is evident that these alternative structures, representing the sequences of the present invention are likewise part of the present invention.

10 The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g. as a restriction fragment) or produced synthetically, which is capable of acting as a point of initiation of synthesis of a primer extension product which is complementary to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g. buffer, salt, temperature and pH) in the presence of nucleotides and an agent for nucleic acid 15 polymerization, such as DNA dependent or RNA dependent polymerase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerization. A typical primer contains at least about 10 nucleotides in length of a sequence substantially complementary or homologous to the target sequence, but somewhat longer primers are preferred. Usually primers contain about 15-26 nucleotides but longer primers may 20 also be employed, especially when the primers contain additional sequences such as a promoter sequence for a particular polymerase.

Normally a set of primers will consist of at least two primers, one 'upstream' and one 'downstream' primer which together define the amplicate (the sequence that will be amplified using said primers).

25 Primarily for the use in transcription based amplification techniques, the oligonucleotides according to the invention may also be linked to a promoter sequence. The term "promoter sequence" defines a region of a nucleic acid sequence that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription by which an RNA transcript is produced. In principle any promoter sequence may be employed 30 for which there is a known and available polymerase that is capable of recognizing the initiation sequence. Known and useful promoters are those that are recognized by certain bacteriophage RNA polymerases such as bacteriophage T3, T7 or SP6. Oligonucleotides linked to a promoter sequence are commonly referred to as "promoter primers".

Oligonucleotides according to the invention are substantially complementary to a sequence of a bacterial EF-Tu mRNA sequence, said oligonucleotide being 10-50 nucleotides in length and comprising at least 10 consecutive nucleotides of one of the sequences depicted in SEQ ID 1-8 or the complementary sequence thereof.

5 Oligonucleotides comprising (a part of) SEQ ID's 1 and 2 have proven to be suitable for the amplification of Ef-Tu mRNA sequences of M.tuberculosis. In the event amplification is carried out with a transcription based amplification technique one of said oligonucleotides may also comprise a promoter sequence. Of course, such "promoter-oligonucleotides" are likewise art of the invention. In the sequence as depicted in SEQ ID 9 the T7 promoter has been linked
10 to the sequence as depicted in SEQ ID 1. In the experimental part of this application these sequences are indicated as TUF15 (SEQ ID 9) and TUF 18 (SEQ ID 2).

The present invention also provides a pair of oligonucleotides for the amplification of M.leprae derived EF-Tu sequences. This pair consists of an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 3 and an oligonucleotide
15 comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 4 respectively.

Again, for use in transcription based methods, one of the oligonucleotides may be linked to a promoter sequence, and an oligonucleotide provided with the T7 promoter sequence is depicted in SEQ ID 10. In the experimental part of the application such a pair is referred to as
20 TUF 20 (SEQ ID 10) and TUF 22 (SEQ ID 4).

The present invention further provides a pair of oligonucleotides that are suitable for the amplification of E.coli derived EF-Tu sequences.

This pair consists of an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 5 and an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 6 respectively.
25

Again, for use in transcription based methods, one of the oligonucleotides may be linked to a promoter sequence, and an oligonucleotide provided with the T7 promoter sequence is depicted in SEQ ID 11. In the experimental part of the application such a pair is referred to as TUF 27 (SEQ ID 11) and TUF 22 (SEQ ID 6).

30 These oligonucleotides are thus especially useful in the assessment of the viability of M.tuberculosis, M.leprae or E.coli.

It is understood that oligonucleotides consisting of the sequences of the present invention may contain minor deletions, additions and/or substitutions of nucleic acid bases, to the extent

that such alterations do not negatively affect the yield or product obtained to a significant degree. Where oligonucleotides according to the present invention are used as probes, the alterations should not result in lowering the hybridization efficiency of the probe.

For example, in case of transcription based amplification techniques, wherein one or more of the primers may be provided with a promoter sequence, the introduction of a purine-rich (= G or A) hybridizing sequence, just after the promoter sequence may have positive effects on the transcription (when there are C's and T's abortive transcription may occur). If no such sequence is available in the target nucleic acid a purine-rich sequence can be inserted in the oligonucleotide just following the last three G residues of the promoter sequence.

The sequences of the present invention are reflected as DNA sequences. The RNA equivalents of these sequences are likewise part of the present invention.

Part of the oligonucleotides according to the invention are particularly suitable for use as a probe in the detection of nucleic acid amplified with a pair of oligonucleotides according to the invention. When used as a probe, said oligonucleotides may be provided with a detectable label. Various labeling moieties are known in the art. Said moiety may, for example, either be a radioactive compound, a detectable enzyme (e.g. horse radish peroxidase (HRP)), a hapten like biotin, or any other moiety capable of generating a detectable signal such as a colorimetric, fluorescent, chemiluminescent or electrochemiluminescent signal.

Hybrids between oligonucleotides according to the invention and (amplified) target nucleic acid may also be detected by other methods known to those skilled in the art. Oligonucleotides according to the invention that are especially suitable as a probe for the detection of Mycobacterial Ef-Tu sequences consist essentially of the sequences as depicted in SEQ ID 7 and 8 (In the experimental part said sequences are depicted as TUF 25 and TUF 26 respectively.).

Together these probes can be used in a sandwich hybridization assay, whereby one can be used as capture probe and the other can be labeled with a detectable label.

A test kit for the detection of Mycobacterial EF-Tu mRNA in a sample is likewise part of the present invention. Such a kit may comprise

a pair of oligonucleotides according to the invention and at least one oligonucleotide comprising a nucleic acid sequence substantially complementary to at least part of the amplified nucleic acid sequence, provided with a detectable label, as well as suitable amplification reagents.

These reagents are for example the suitable enzymes for carrying out the amplification reaction. A kit, adapted for use with NASBA for example, may contain suitable amounts of reverse transcriptase, RNase H and T7 RNA polymerase. Said enzymes may be present in the kit in a buffered solution but can likewise be provided as a lyophilized composition, for 5 example, a lyophilized spherical particle. Such lyophilized particles have been disclosed in PCT appl. no. EP95/01268. The kit may further be furnished with buffer compositions, suitable for carrying out an amplification reaction. Said buffers may be optimized for the particular amplification technique for which the kit is intended as well as for use with the particular oligonucleotides that are provided with the kit. In transcription based amplification techniques, 10 such as NASBA, said buffers may contain, for example, DMSO, which enhances the amplification reaction (as is disclosed in PCT appl. no. US90/04733).

Furthermore the kit may be provided with an internal control as a check on the amplification procedure and to prevent the occurrence of false negative test results due to failures in the amplification procedure. The use of internal controls in transcription based 15 amplification techniques is described in PCT appl. no. EP 93/02248. An optimal control sequence is selected in such a way that it will not compete with the target nucleic acid in the amplification reaction. Kits may also contain reagents for the isolation of nucleic acid from biological specimen prior to amplification. A suitable method for the isolation of nucleic acid is disclosed in EP389063.

20

BRIEF DESCRIPTION OF THE FIGURES:

- Figure 1a: The analytical sensitivity of NASBA using in vitro produced mycobacterial EF-Tu 25 RNA.
Figure 1b: The analytical sensitivity of NASBA using in vitro produced mycobacterial EF-Tu RNA.
Figure 2: Specificity of the M.tuberculosis NASBA.
Figure 3: Specificity of the M.leprae NASBA.
30 Figure 4: NASBA for viability assessment.

EXAMPLES:

Example 1:

5

Selection of primers and probes for the amplification of EF-Tu mRNA of *M.tuberculosis*, *M.leprae* and *E.coli*:

Sources of RNA. Table 1 shows the sources of RNAs that were used in the experiments
10 described in this and the following examples. The cultivable mycobacteria were grown on Löwenstein-Jensen slants for 2-3 weeks. *M.leprae* was isolated from spleen tissue of an experimentally infected armadillo (*Dasypus novemcinctus* Linn.), as recommended by the World Health Organization [WHO Expert Committee on Leprosy. (1988) Sixth Report Tech. Rep. Ser. 8. 768. World Health Organization, Geneva.]. Other bacteria which might be found in
15 human and/or animal samples or which are closely related to mycobacteria (see Table 1), were used for controls. For *Actinomyces israelii* lyophilized bacteria were used. The strains used for the production of in vitro RNA are described below.

Table 1. Sources of RNA and specificity of NASBA

	Species	Strain/source	Origin	ECL signal M.tuberculosis primers	ECL signal M.leprae primers
1.	Mycobacterium africanum	myC 5544	RIVM	20810	+
2.	Mycobacterium avium	3875 (serovar 2)	RIVM	1523 1344	- 1031 1217
3.	Mycobacterium bovis	8316	RIVM	1E+08 2109782	+
4.	Mycobacterium bovis	ATCC 19210	RIVM	36497 246050	970 1006
5.	Mycobacterium bovis BCG	ATCC 35733	SSI	117186 472834	+
6.	Mycobacterium intracellulare	IWGMT3 (serovar 4)	RIVM	1322 1310	1089 1069
7.	Mycobacterium kansasi	1012	RIVM	1418 1341	- 920 1052
8.	Mycobacterium leprae	Armadillo isolate	KIT	1001 3592	- 1060 1135
9.	Mycobacterium paratuberculosis	138601-24	CDI	1146 1125	- 1543836 1E+08 851 1019

	Species	Strain/source	Origin	ECL signal M.tuberculosis primers	ECL signal M.leprae primers
10.	<i>Mycobacterium scrofulaceum</i>	3442	RIVM	1143 1167	- 870 1097
11.	<i>Mycobacterium smegmatis</i>	ATCC 14468	RIVM	1365 1289	- 954 1058
12.	<i>Mycobacterium tuberculosis</i>	4514	RIVM	401796 24398	+ 902 1914
13.	<i>Mycobacterium tuberculosis</i>	H37RV	RIVM	144963	+
14.	<i>Mycobacterium tuberculosis</i>	M. tub 193	KIT	1417077	+
15.	<i>Actinomyces israelii</i>	103.62	CBS	886 1104	- 972 1974
16.	<i>Corynebacterium beaufanti</i>	Clinical isolate	AMC	1048 1029	- 966 1818
17.	<i>Corynebacterium J.K.</i>	Clinical isolate	AMC	1108 1113	- 966 2082
18.	<i>Escherichia coli</i>	Clinical isolate	AMC	1129 1062	- 884 2219
18a.	<i>Escherichia coli</i>	INVαF'	Invitrogen (K2000-01)	NT	NT

	Species	Strain/source	Origin	ECL signal M.tuberculosis primers	ECL signal M.leprae primers
19.	<i>Haemophilus influenza</i>	Clinical isolate	AMC	1097 1113	- 2085
20.	<i>Klebsiella pneumoniae</i>	Clinical isolate	AMC	1137 1117	- 1022 1957
21.	<i>Legionella pneumophila</i>	Clinical isolate	AMC	1017 954	- 999 1914
22.	<i>Nocardia asteroides</i>	Clinical isolate	AMC	970 1037	- 1239 2079
23.	<i>Pseudomonas aeruginosa</i>	Clinical isolate	AMC	981 937	- 965 1757
24.	<i>Staphylococcus aureus</i>	Clinical isolate	AMC	932 1006	- 967 2214
25.	<i>Streptococcus pneumoniae</i>	Clinical isolate	AMC	909 985	- 977 2081
26.	<i>Homo sapiens</i>	Placenta	AMC	1041	- 2073
27.	<i>Homo sapiens</i>	Placenta Lot no. BD 41091901	Pharmacia	991	- 1986

	Species	Strain/source	Origin	ECL signal M.tuberculosis primers	ECL signal <i>M.leprae</i> primers
28.	In Vitro RNA 1054 Mycobacterium tuberculosis H37RV	764 bp in PCR-II vector pTHT 1054 (plasmid) S1418 (strain INVaF')	KIT	409019 1E+08	+
29.	In Vitro RNA 1055 Mycobacterium leprae Armadillo isolate	764 bp in PCR-II vector pTHT 1055 (plasmid) S1419 (strain INVaF')	KIT	992 764	-
30.	In Vitro RNA 1057 Escherichia coli Clinical isolate	764 bp in PCR-II vector pTHT 1057 (plasmid) S1420 (strain INVaF')	KIT	986 984	-
31.	Water purified according to Boom et al. 1991 [9]		Baker	1194 960	-
32.	Water		Baker	995 678	-
				612	-

AMC= Academic Medical Centre, Amsterdam, The Netherlands; CBS= Netherlands Culture Collections of Micro-Organisms, Baarn, The Netherlands; CDI= Central Veterinary Institute, Lelystad, The Netherlands; KIT= Royal Tropical Institute, Amsterdam, The

Netherlands; RIVM= National Institute of Public Health and Environment, Bilthoven, The Netherlands; SSI= Statens Serum Institute, Copenhagen, Denmark; ATCC= American Type Culture Collection, Rockville, USA.

Nucleic acid (NA) isolation. To perform the experiments described in all examples nucleic acids were isolated as we described previously [Vliet, G.M.E. van der, Schepers, P., Schukkink, R.A.F., Gemen, B. van and Klatser, P.R. (1994) Assessment of mycobacterial viability by RNA amplification. *Antimicrob. Agents Chemother.* 38, 1959-1965.]. In summary, all bacterial strains were adjusted to the turbidity equivalent of no. 4 McFarland barium sulphate nephelometer standard as described earlier [Verstijnen, C. P. H. J., H. M. Ly, K. Polman, C. Richter, S. P. Smits, S. Y. Maselle, P. Peerbooms, D. Rienthong, N. Montreewasuwat, S. Koanjanart, D. D. Trach, S. Kuijper, and A. H. J. Kolk (1991) Enzyme-linked immunosorbent assay using monoclonal antibodies for identification of mycobacteria from early cultures. *J. Clin. Microbiol.* 29, 1372-1375.]. Fifty μ l of the diluted samples contained approximately 10^5 viable mycobacteria as determined by counting the number of colony-forming units (not done for *M.leprae*). This volume was used for lysis and NA-isolation according to "protocol Y/SC" described by Boom et al. (1990) [Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen and J. Van der Noordaa (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28, 495-503.]. The NA were eluted from the silica with 50 μ l or 100 μ l RNase-free H₂O and stored at -20°C.

Human placental NA were isolated in a similar fashion (kindly provided by Dr. H. Smits, Department of Virology, Academic Medical Hospital, Amsterdam, The Netherlands). Two μ l containing 270 ng NA was tested. This amount corresponds to approximately 4 x 10^5 diploid human cells. Another batch of human NA was obtained commercially from Pharmacia (Uppsala, Sweden).

25 Example 2:

Sensitivity and specificity of NASBA in the amplification of EF-Tu mRNA of *M.tuberculosis*, *M.leprae* and *E.coli*:

30 Selection of primers and probes. The primers and probes used in this study are listed in Table 2.

Table 2. Primers and probes

Primer/ probe	Origin	Position	Sequence (5'-3')
TUF 4	MTTUF	1302-1284	GTR CGG AAG TAG AAC TGC G
TUF 7	MTTUF	539-560	GAC IIC CCI GGI CAC GCC GAC T
TUF 3F	MTTUF	917-936	Fluorescein-GAC AAG CCG TTC CTG MTG CC
TUF 4F	MTTUF	1302-1284	Fluorescein-GTR CGG AAG TAG AAC TGC G
TUF 8F	MTTUF	936-917	Fluorescein-GGC AKC AGG AAC GGC TTG TC
TUF 15	MTTUF	1057-1039	aat tct aat acg act cac tat agg gAG AGC TTG GTG GAT GGG CGA
TUF 18	MTTUF	855-875	CCT CTG TCG AGG AAC TGA TGA
TUF 20	MLTUF	977-958	aat tct aat acg act cac tat agg gAG AGG GTC GTC TGA CGA ATG CCG A
TUF 22	MLTUF	781-801	AGT CTG TCA CAC AGT TGA TGG
TUF 24	MLTUF	862-843	Digoxigenin-GGC ATC AGG AAC GGC TTG TC
	MTTUF	936-917	
TUF 25	MLTUF	920-937	Biotin-GCG CGG CGT GGT CAA CGT@
	MTTUF	994-1011	
TUF 26	MLTUF	940-957	Ruthenium-ACG AGG AAG TTG AGA TCG
	MTTUF	1014-1031	
TUF 27	ECTUF	756-737	aat tct aat acg act cac tat agg gAG AGC TGA GTC TCT TTG ATA CCA A
TUF 28	ECTUF	560-580	CGA AAA TCC TGG AAC TGG CTG

TUF 29	ECTUF	699-716	Biotin-ACG CGG TAT CAT CAA AGT
TUF 30	ECTUF	719-736	Ruthenium-GTG AAG AAG TTG AAA TCG

Abbreviations:

- 5 ECTUF = *E. coli* EF-Tu gene sequence (GenBank accession number J01717)
 MTTUF = *M. tuberculosis* EF-Tu gene sequence (GenBank accession number X63539)
 MLTUF = *M. leprae* EF-Tu gene sequence (GenBank accession number D13869)
 I = Inosine, M=[A,C], K = [G,T], R = [A,G]
 @ The sequence shown is homologous to MLTUF and not to MTTUF (where the underlined G is an A).
 10 TUF 15, 20 and 27 include the T7 promoter sequence which is shown in lower case.
 The promoter sequence is followed by a purine rich region (AGAG) which is shown in italics.

The published nucleotide sequences of the EF-Tu genes of *M.tuberculosis* (GenBank accession number X63539), *M.leprae* (GenBank accession number D13869), *E. coli* (GenBank accession number J01717), *Micrococcus luteus* (GenBank accession number M17788) and *Streptomyces coelicolor* (GenBank accession number X77039) were aligned
5 using the software programme GCG (National Institute of Health, USA) installed on appropriate hardware. Degenerative primers TUF4 and TUF7 (Table 2) were selected to amplify a 764 bp DNA fragment of the EF-Tu genes by PCR from all organisms listed in Table 1. PCR was performed using 75 mM Tris-HCl, pH9.0, 20 mM (NH₄)₂SO₄, 0.01% (v/v) Tween 20, 4 mM MgCl₂, 0.2 mM of each dNTP, 125 ng of each primer and 1 U/reaction of
10 Goldstar DNA polymerase (Eurogentec, Belgium). PCR was initiated by incubation at 94 °C for 3 min followed by 35 cycles of 1.15 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The generated PCR fragments of the DNA of the organisms listed in Table 1, except *C.belfanti*, *H.influenza*, *S.aureus*, *S.pneumoniae* and *H. sapiens* were purified using a MicroSpin Sephadryl 300 HR column (Pharmacia) to remove excess of primers. The purified
15 fragments were sequenced directly using primers TUF 3F, 4F and 8F by applying the Autocycle Sequencing Kit (Pharmacia) and the A.L.F. automatic DNA sequencer (Pharmacia). Sequences were edited by using the DNASIS software programme (Pharmacia) and clustal alignments were then made using the PCGene software programme.
20

Selection of primers and probes. Table 3 shows the seven variable regions of the EF-Tu sequences which were defined based on the clustal alignments. From these variable regions it is possible to select primers and probes enabling species-specific detection of EF-Tu mRNA of different organisms (Table 4).

25 Table 5 shows the clustal alignments of two (region IV and VI) of the seven variable regions of the EF-Tu sequence on the basis of which the primers and probes were selected for specific amplification of *M.tuberculosis*, *M.leprae* and *E.coli* EF-Tu mRNA through NASBA. The criteria to select these two areas were: the availability of species-specific primers, a genus-specific probe and a length of ± 200 nucleotides of the fragment to be
30 amplified.

Table 3. Seven variable regions on the EF-Tu sequences

Variable Region	Position on the EF-Tu sequence (in bp) of <i>Mycobacterium tuberculosis</i> AC X63539
I.	673- 703
II.	774- 793
III.	820- 823
IV.	852- 871
V.	955- 970
VI.	1039-1051
VII.	1123-1142

Table 4.
 Parts of the EF-Tu mRNA variable sequences which can be used
 for the selection of primers and/or probes
 enabling species-specific detection of EF-Tu mRNA from different organisms.

Region	Sequence	Specific for
I.	AGTGGGTGTCACATCCCTGGTAGCGCTG	Mycobacterium tuberculosis complex
	GGTGGGTGTAACCTAACATCCTGGTCGCACCT	Mycobacterium leprae
	GGTGGGTGACATCCTGGCGGCTG	Mycobacterium avium and Mycobacterium paratuberculosis
	GGTGGCGTGCCGCCCTGCTCGTGGCCCTG	Micrococcus luteus
	GGTGGCGTTCCGTACATCGTGGCGCCCTG	Streptomyces coelicolor
	GGTGGCGTTCCCTAACATCCTCGTTGCTCTT	Corynebacterium glutamicum
	GGTGGCGTTCCCTAACATCCTGGTTGCACCTG	Corynebacterium J.K.
	GGTAGGGGTCCGTACATCATCGTGGTCCCTG	Escherichia coli, Nocardia asteroides and Klebsiella pneumoniae
	GGTAGGGCGTTCCCTAACATCGTGGTCCCTG	Pseudomonas aeruginosa
II.	CTGCCCAAGGAATTGACGAG	Mycobacterium tuberculosis complex and Mycobacterium leprae
	CCGCCAGGGAGTTCGACGAG	Mycobacterium avium and Mycobacterium paratuberculosis

Region	Sequence	Specific for
	CCGCCAGGAGTTC	Mycobacterium intracellulare
	TCTCAGTACGACCTCCGGGC	Escherichia coli
	TCTCAGTACGATTCCGGGC	Nocardia asteroides and Klebsiella pneumoniae
	CCTCCAGGAGCTTCGACGTC	Micrococcus luteus
	CTCCGAGTAGGAGTCCCCGGGAC	Streptomyces coelicolor
	CTGAGCAGGAACTACGAC	Corynebacterium glutamicum
	AACACCTACGACTTCCGGGC	Pseudomonas aeruginosa
III.	GCTC	Mycobacterium tuberculosis complex, Mycobacterium avium, Mycobacterium paratuberculosis and Mycobacterium intracellulare
	ATTG	Mycobacterium leprae
	TCTG	Micrococcus luteus, Streptomyces coelicolor, Corynebacterium glutamicum, Escherichia coli, Nocardia asteroides, Klebsiella pneumoniae and Actinomyces israelii
	GCTG	Pseudomonas aeruginosa
IV.	TTGCTCTGTGAGGAACGTGATGA	Mycobacterium tuberculosis complex
	TTGCTCTGTGAGGAACGTGATGA	Mycobacterium tuberculosis (AC X63539)
	TCGAGTCTGTCAACACAGTTGATGG	Mycobacterium leprae
	TCAAGTCGGTGGAGGACCTCATGG	Micrococcus luteus

Region	Sequence	Specific for
	GCAACTCGGTCTGGAGCTCATGA	Streptomyces coelicolor
	GCAAGCAGATCCTTGAGCTCATGC	Corynebacterium glutamicum
	TGGAGTCCGTCGAGCAG	Mycobacterium avium and Mycobacterium paratuberculosis
	AAGGAAAAATCCTGGAACTGGCTGGC	Escherichia coli
	AAGCGAAAATC	Nocardia asteroides and Klebsiella pneumoniae
V.	CATTACCGGGCGGGGA	Mycobacterium tuberculosis
	TATCACCGGGTCTGGC	Mycobacterium leprae
VI.	TCGGCATTGCCCATCGACCACCAAG	Mycobacterium tuberculosis complex
	TCGGCATTGGTCAGACGACCACCAAG	Mycobacterium leprae
	TCGGCATCCGCCGACCAGCACCACCAAG	Mycobacterium intracellulare
	TCGGCATCCGCCGTCCAGCACCACCAAG	Mycobacterium avium and Mycobacterium paratuberculosis
	TCGGCATCCGGAGGACCCACCAAG	Mycobacterium smegmatis
	TTGGTATCAAAGAGACTCAGAAGT	Escherichia coli
	TTGGTATCAAAGAGACCGGGAAAA	Klebsiella pneumoniae
	TCGGCATCAAGGGGACCCACCAAGA	Pseudomonas aeruginosa
	CATTAAGCCCCAGCACCACCAAGA	Mycobacterium scrofulaceum
	TCGGCATCCGTCCGACACCCACCAAGA	Mycobacterium kansassii
VII.	TGGTTTGCTGCTGGGGCG	Mycobacterium tuberculosis complex
	TGGTCTGTTGGCTGGCA	Mycobacterium leprae
	CGGTCTGCTGCTGGTGGTA	Mycobacterium intracellulare, Mycobacterium avium and

Region	Sequence	Specific for
	TGGTAACCTGCTGGCTGGCA	Mycobacterium paratuberculosis
	CGGTCTGTTGCTCCGGCCA	Pseudomonas aeruginosa
	CGGGTCTGTTGCTGGCTGGTG	Mycobacterium scrofulaceum
	AGGTGTTCTGCTGGGGTA	Mycobacterium kansasii
	AGGTGTTCTGCTGGTGGTA	Escherichia coli
		Klebsiella pneumoniae

Table 5.

Two clustal alignments (region IV and VI) of the variable regions of the EF-Tu sequence

Species	Number AC or Table 1	Sequence (Region IV)
Mycobacterium tuberculosis	X63539	TTGCCTCTGTC-GAGGAA--CT-GATGA
Mycobacterium leprae	D13869	TCGAGTCTGTC-ACACAG--TT-GATGG
Micrococcus luteus	M17788	TCAA---GTCCGTGAGGACCT-CATGG
Streptomyces coelicolor	X77039	GCAAACTCGGTCC-TCGAG---CT-CATGA
Corynebacterium glutamicum	X77034	GCAAAG-CAGATC-CTTGAGGC-T-CATGC
Mycobacterium leprae	8	TCGAGTCTGTCA--AC
Mycobacterium tuberculosis H37Rv	13	TTG---C-TCTGTCGAGGAAC-T-GAT
Mycobacterium bovis	3,4	TTG---C-TCTGTCGAGGAAC-T-GAT
Mycobacterium bovis BCG	5	TTG---C-TCTGTCGAGGAAC-T-GAT
Mycobacterium avium	2	TGG---A-GTCCGTGAGCAGCT-GAT
Mycobacterium paratuberculosis	9	TGG---A-GTCCGTGAGCAG
Mycobacterium tuberculosis	12	TTG---C-TCTGTCGAGG
Escherichia coli	J01717	AAGCGAAAATC---CTGAAACTGGCTGGC
Escherichia coli	18	AAGCGAAAATC---CTGAAACTG
Nocardia asteroides	22	AAGCGAAAATC

<i>Mycobacterium leprae</i>	D13869	TCGGCATTGGTCAGA-CGACCAAG
<i>Mycobacterium tuberculosis</i>	X63539	TCGGCATTGCCCAT-CGACCAAG
<i>Mycobacterium bovis</i>	4	TCGGCATTGCCCAT-CGACCAAG
<i>Mycobacterium leprae</i>	8	TCGGCATTGGTCAGA-CGACCAAG
<i>Mycobacterium intracellulare</i>	6	TCGGCATTGCCGA-CCAGCACCAAG
<i>Mycobacterium bovis BC G</i>	5	TCGGCATTGCCCAT-CGACCAAG
<i>Mycobacterium tuberculosis</i>	12	TCGGCATTGCCCAT-CGACCAAG
<i>Mycobacterium tuberculosis H37Rv</i>	13	TCGGCATTGCCCAT-CGACCAAG
<i>Mycobacterium avium</i>	2	TCGGCATTGCCGT-CCAGCACCAAG
<i>Mycobacterium paratuberculosis</i>	9	TCGGCATTGCCGT-CCAGCACCAAG
<i>Mycobacterium smegmatis</i>	11	TCGGCATTGCCGGA-GACCAAG
<i>Mycobacterium scrofulaceum</i>	10	TCAGCATTAAAGGCCGC-CCAGCACCAAG
<i>Mycobacterium kansasii</i>	7	TCGGCATTGGTCGA-CACCA-CAG
<i>Escherichia coli</i>	18	TTGGTAT---CAAAGAGA-CTCAGAAGT
<i>Klebsiella pneumoniae</i>	20	TTGGTAT---CAAAGAGA-CCGGAAAA
<i>Pseudomonas aeruginosa</i>	23	TCGGCATT---CAAGGGGA-CCACCAAGA

Based on these alignments species-specific primers for the use in NASBA were selected for M.tuberculosis (TUF 15 and 18), for M.leprae (TUF 20 and 22) and for E.coli (TUF 27 and 28). A generic capture probe TUF 25 and detection probe TUF 26 were chosen for the detection of the mycobacterial RNA amplicons by electrochemiluminescence (ECL). Probe TUF 25 is homologous to the EF-Tu sequence of M.leprae and not to that of M.tuberculosis; the difference is one nucleotide (G→A; see Table 2). However, this difference did not affect the detection of amplicons generated from RNA originating from M.tuberculosis. For the detection of E.coli RNA amplicons we used the specific capture and detection probes TUF 29 and TUF 30, respectively.

In region IV one nucleotide difference was found with the published sequence of EF-Tu of M.tuberculosis [GenBank accession number X63539]: in none of the strains belonging to the M.tuberculosis complex a C at position 855 was found. However, the primer position was chosen in such a way that this discrepancy would not influence the amplification reaction.

15

Example 3:

Specificity and sensitivity testing of E.coli by amplification of EF-Tu mRNA.

20

Isolation of in vitro RNA.

A 764 bp fragment of the EF-Tu gene of M.tuberculosis H37Rv, M.leprae and E.coli (Table 1) was amplified via PCR using the primer set TUF 4 and TUF 7 (Table 2), as described above. The amplified product was cloned into the pCR II vector using the TA-cloning kit (Invitrogen) following the manufacturer's instructions. Selection of the appropriate clone with the insert in the correct orientation, size and specificity was determined by restriction enzyme analysis and PCR.

After linearization of the plasmid with the restriction enzymes Bam HI and Hind III, the cloned insert within the polylinker region was transcribed from the T7 promoter site using the SP6/T7 transcription kit (Boehringer, Mannheim). The DNA template was removed by digestion with RQI DNase (Promega) and the RNA was purified by the RNeasy kit from Qiagen according the manufacturer's protocol. Purity was checked by agarose gel electrophoresis and Northern blotting with digoxigenine-labelled probe TUF 24 according to standard procedures. The in vitro RNA was quantified by measuring the extinction at 260 nm.

RNA Amplification.

A 203, 197 and 198 nucleotide fragment of the EF-Tu mRNA of *M.tuberculosis*, *M.leprae* and *E.coli*, respectively, was amplified by the NASBA technique essentially as described before [Vliet, G.M.E. van der, Schepers, P., Schukkink, R.A.F., Gemen, B. van 5 and Klatser, P.R. (1994) Assessment of mycobacterial viability by RNA amplification. Antimicrob. Agents Chemother. 38, 1959-1965.]. The 20 µl reaction mix was composed of 40 mM Tris-HCl, pH 8.5, 12 mM MgCl₂, 70 mM KCl, 5 mM DTT, 1.5 M sorbitol, 2.1 mg BSA, 1 mM of each dNTP, 2 mM of ATP, CTP, UTP, 1.5 mM of GTP and 0.5 mM ITP, 15%: 10 (v/v) DMSO, 0.2 mM Primer 1 and Primer 2, 0.08 U RNase H, 32 U T7 RNA polymerase, 6.4 U AMV-RT polymerase and the RNA target. Isothermal amplification of the RNA target was performed by incubation of these samples at 41 °C for 2.0 h.

Detection of the amplified RNA was done by in-solution hybridization in the ECL detection assay, as described previously [Gemen, B. van, Beuningen, R. van, Nabbe, A., Strijp, D. van, Jurriaans, S., Lens, P., Kievits, T. (1994) A one-tube quantitative HIV-I RNA 15 NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labelled probes. J. Virol. Methods, 49, 1] with minor modifications: 5 µl of NASBA amplified RNA was either diluted 20-fold in RNase-free water or used undiluted. A 5'-biotinylated probe was used to capture the NASBA product. The detection probe used was tris [2,2-bipyridine] ruthenium [II] complex labelled. This label emits light as a result of chemical reactions 20 taking place at the surface of an electrode. The cutoff value was set at 3000. The ECL detection assay was measured on a scale of 0 to 10⁸.

Controls for amplification.

Negative controls (water only) were included in each experiment in order to check for 25 carry-over contamination during NA-extraction and amplification. These control samples were extracted and amplified by NASBA in the same manner as described above.

Sensitivity and specificity of the mycobacteria NASBA.

For determination of the sensitivity of the NASBA, *M.tuberculosis* ATCC 35801 was 30 grown in liquid Tween/Albumin medium at 37 °C. The concentration at the start of the culture was 8.10⁶ bacteria/ml. Growth of the bacteria was monitored by measuring the extinction at 420 nm. After 13 days of culture a sample was taken and diluted in lysis buffer [Boom, R., C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen and J. Van der Noordaa (1990). Rapid and simple method for purification of nucleic acids. J. Clin. 35 Microbiol. 28, 495-503.]. Serial dilutions were made in lysis buffer, RNA purified [Boom, R.,

C.J.A. Sol, M.M.M. Salimans, C.L. Jansen, P.M.E. Wertheim-Van Dillen and J. Van der Noordaa (1990). Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* 28, 495-503.] and each dilution was tested in NASBA to determine the highest dilution still giving a positive NASBA signal. In addition the analytical sensitivity of NASBA 5 was determined using serial dilutions of in vitro RNA (see above). The specificity of NASBA was determined using the purified RNA from different organisms (Table 1, see above).

Sensitivity and specificity of the E.coli NASBA.

E.coli was grown in Luria Broth (LB) liquid medium at 37 °C for 18 h. The suspension 10 thus obtained was inoculated in fresh medium (1:200)(OD 600nm = 0.015) and incubated at 37 °C in a gyrotory shaker for 3 h and 15 min (OD 600nm = 0.430). A serial dilution was made and mRNA was purified as described above. In addition, the same dilutions were plated onto LB agar plates which were incubated at 37 °C for 18 h after which colonies were counted.

15 The analytical sensitivity of NASBA was determined using serial dilutions of in vitro RNA (see above).

Results:

Sensitivity of NASBA. The analytical sensitivity of NASBA using in vitro produced EF-Tu RNA is illustrated in Figure 1a and 1b. Both the *M.tuberculosis* NASBA and the *M.leprae* NASBA had a detection of 50 molecules of RNA (Fig 1a). The detection limit of the *M.tuberculosis* NASBA when using bacteria as starting material for detection was 12,000 (result not shown).

25 The analytical sensitivity of the E.coli NASBA was shown to be 100 molecules (Fig. 1b). The detection limit of the E.coli NASBA when using bacteria as starting material for detection was 0.4 (result not shown).

Specificity of NASBA. The specificity of the *M.tuberculosis* NASBA and the *M.leprae* NASBA is illustrated in Figures 2 and 3, respectively.

30 The *M.tuberculosis* NASBA showed specificity for the RNA purified from bacteria belonging to the *M.tuberculosis* complex only. Furthermore, as illustrated in Figure 2, the *M.tuberculosis* NASBA showed a positive reaction when homologous in vitro produced EF-Tu RNA was used as target.

35 The *M.leprae* NASBA showed specificity for *M.leprae* RNA only and its homologous in vitro produced RNA.

Example 4:

5 Viability testing of E.coli.

E.coli was grown in LB liquid medium at 37 °C for 18 h. The suspension thus obtained was inoculated in fresh medium (1:1000)(OD 600nm = 0.001) and incubated at 37 °C in a gyrotory shaker for 4 h. The suspension was then divided in two equal parts. To one 10 part a cocktail of antibiotics was added to kill the bacteria: ampicillin, rifampicin and kanamycin (each 50 µg/ml); the other part was left untouched. Both were incubated at 37 °C for another 3 h and 30 min. Every 30 min the OD at 600 nm was monitored and a 100 sample from each culture was taken and added to 900 lysis buffer; mRNA was purified as described above (9). In addition, viability of the E.coli bacteria was monitored by overlaying 15 a sample (100) onto LB agar plates. Colonies were counted after 18 h incubation at 37 °C.

The NASBA signals increased and reached its maximum level when E.coli was left to grow untouched (Figure 4). However, when antibiotics were added to the exponentially growing E.coli culture, the NASBA signal decreased 1 h after the addition of the antibiotics. This drop in mRNA concentration as measured by NASBA was coinciding with a decrease 20 in the number of viable counts (Figure 4).

CLAIMS:

1. Method for the assessment of bacterial viability whereby mRNA coding for the elongation factor EF-Tu is used as a target in a nucleic acid amplification reaction and
5 the presence and/or amount of said mRNA is determined.
2. Method according to claim 1, wherein said nucleic acid amplification reaction is a transcription based amplification reaction.
- 10 3. Method according to claim 2, wherein the transcription based amplification method is NASBA.
4. Method according to any of claims 1-3, wherein the amplified mRNA is detected with a complementary labeled probe.
15
5. Method according to claim 4, wherein the probe is provided with an electro-chemiluminescent label.
6. Method according any of the preceding claims wherein the bacteria are Mycobacteria.
20
7. Method according to claim 6, wherein the Mycobacteriae species are M.tuberculosis or M.leprae.
8. Oligonucleotide substantially complementary to a sequence of a bacterial EF-Tu
25 mRNA sequence, said oligonucleotide being 10-50 nucleotides in length and comprising at least 10 consecutive nucleotides of one of the sequences depicted in SEQ ID 1-8 or the complementary sequence thereof.
9. Pair of oligonucleotides for the amplification of a sequence in the EF-Tu gene of
30 M.tuberculosis, said pair consisting of an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 1 and an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 2 respectively.

10. Pair of oligonucleotides for the amplification of a sequence in the EF-Tu gene of M.leprae, said pair consisting of an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 3 and an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ
5 ID 4 respectively.
11. Pair of oligonucleotides for the amplification of a sequence in the EF-Tu gene of E.coli, said pair consisting of an oligonucleotide comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 5 and an oligonucleotide
10 comprising at least 10 consecutive nucleotides of the sequence as depicted in SEQ ID 6 respectively.
12. Method for the assessment of the viability of M.tuberculosis, M.leprae or E.coli wherein a pair of oligonucleotides according to claim 9, 10 or 11 is used respectively
15 for amplifying Ef-Tu mRNA and the presence and/or amount of amplified nucleic acid is detected.
13. Method according to claim 12, wherein the viability of M.tuberculosis or M.leprae is assessed and detection of amplified nucleic acid is carried out using a sandwich hybridization assay with a capture probe having at least 10 consecutive nucleotides of a sequence as depicted in SEQ ID 7 and a labeled detection probe having at least 10 consecutive nucleotides of the sequence depicted in SEQ ID 8.
20
14. Method according to claim 12, wherein the viability of E.coli is assessed and detection of amplified nucleic acid is carried out using a sandwich hybridization assay with a capture probe having at least 10 consecutive nucleotides of a sequence as depicted in SEQ ID 12 and a labeled detection probe having at least 10 consecutive nucleotides of the sequence depicted in SEQ ID 13.
25
- 30 15. Test kit for the detection of Mycobacterial EF-Tu mRNA in a sample comprising:
 - a pair of oligonucleotides according to claim 9 or 10 ,
 - at least one oligonucleotide comprising a nucleic acid sequence substantially complementary to at least part of the amplified nucleic acid sequence , provided with a detectable label
 - suitable amplification reagents.

- 16 Test kit for the detection of E.coli bacterial EF-Tu mRNA in a sample comprising:
- a pair of oligonucleotides according to claim 11,
 - at least one oligonucleotide comprising a nucleic acid sequence substantially complementary to at least part of the amplified nucleic acid sequence , provided with a detectable label
 - suitable amplification reagents.

10

Figure 1a: The analytical sensitivity of NASBA using in vitro produced mycobacterial EF-Tu RNA

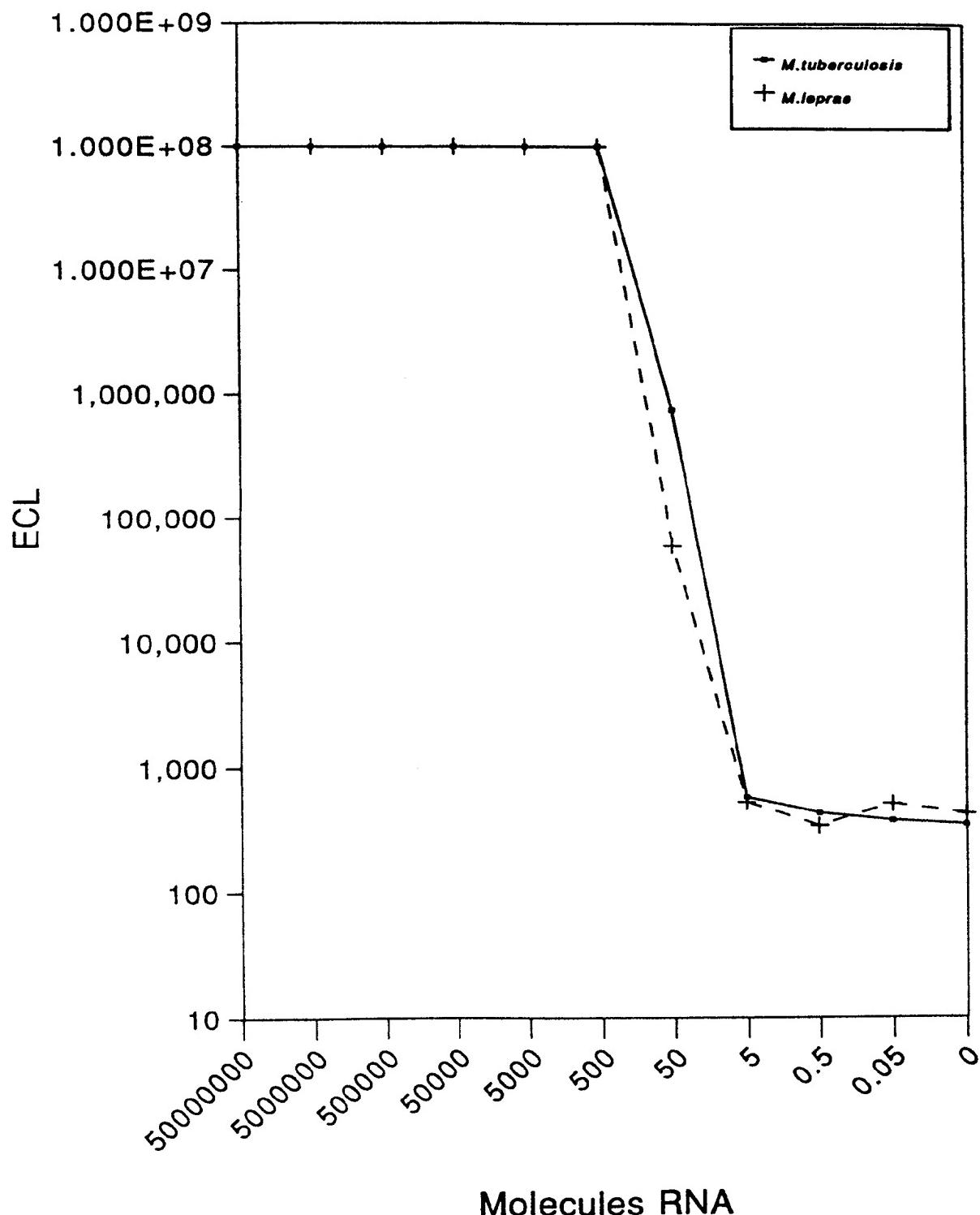


Figure 1b: The analytical sensitivity of NASBA using in vitro produced *E.coli* EF-Tu RNA

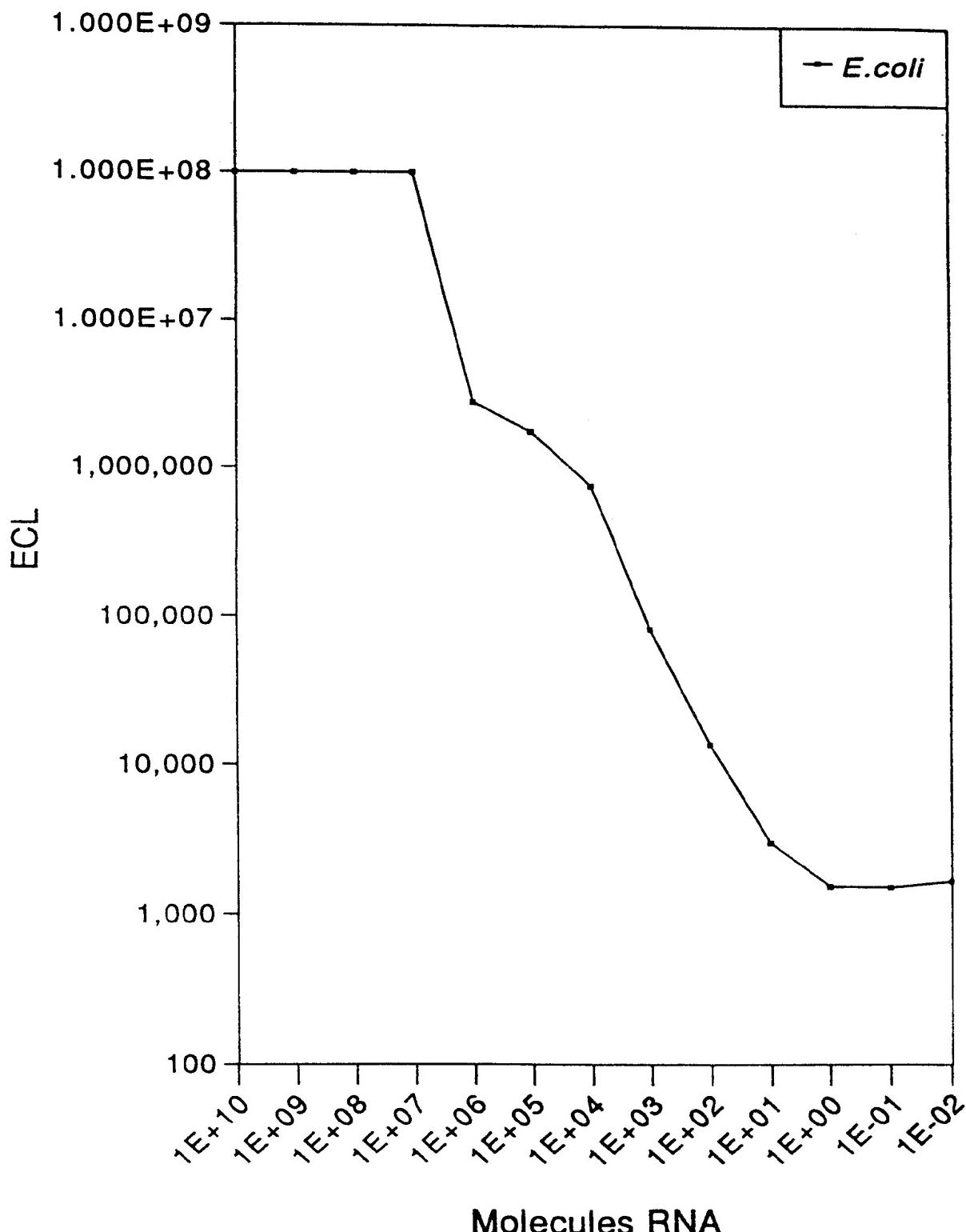


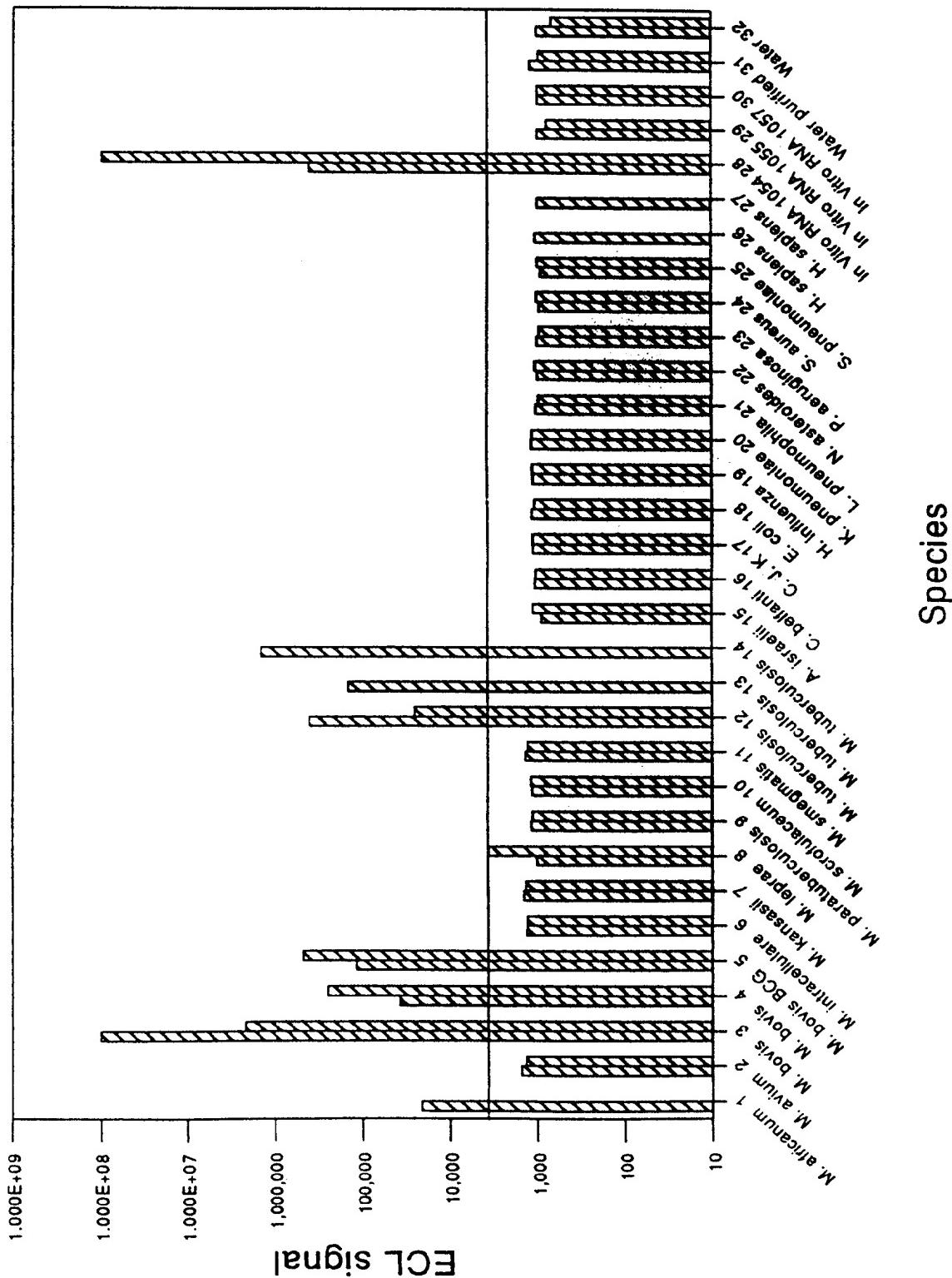
Figure 2: Specificity of the *M. tuberculosis* NASBA

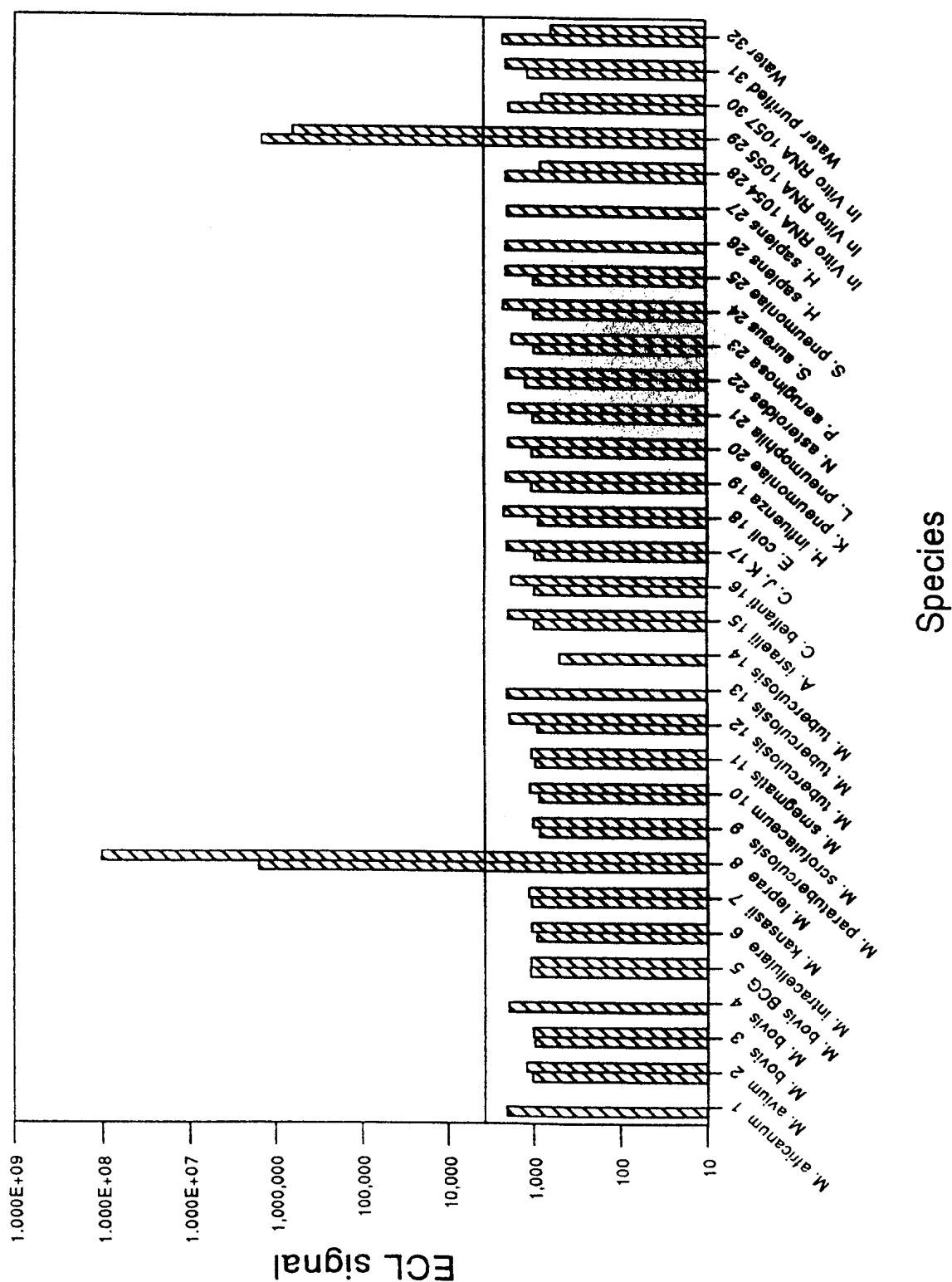
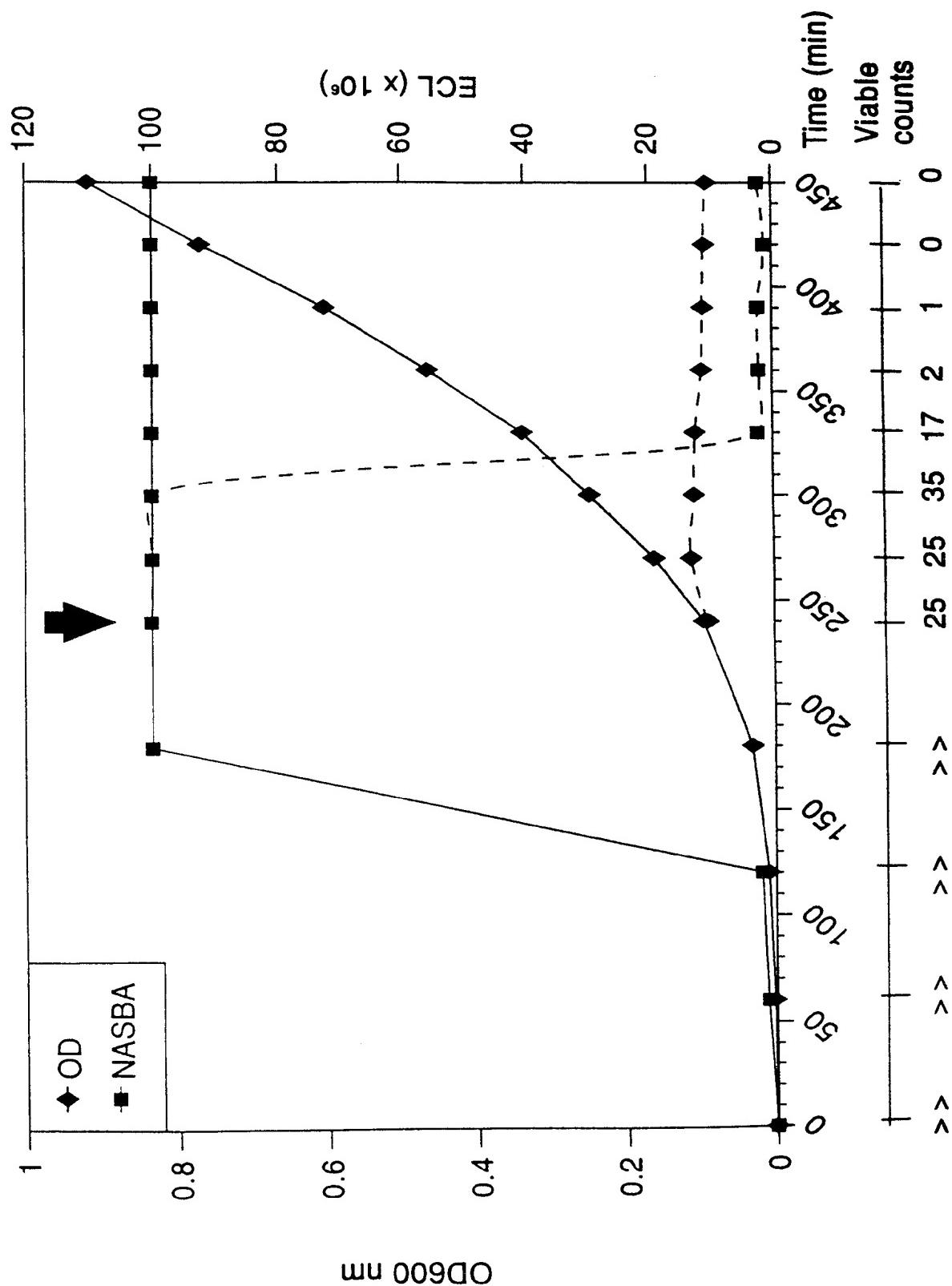
Figure 3: Specificity of the *M. leprae* NASBA

Figure 4: NASBA for viability assessment



SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: Akzo Nobel N.V.
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- 10 (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM
- (G) TELEPHONE: 0412 666379
- (H) TELEFAX: 0412 650592

15 (ii) TITLE OF INVENTION: EF-TU mRNA as a marker for viability of bacteria

(iii) NUMBER OF SEQUENCES: 95

(iv) COMPUTER READABLE FORM:

- 20
- (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- 30 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AATTCTAATA CGACTCACTA TAGGGAGAGC TTGGTGGTCG ATGGCGA

48

5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCTCTGTCGA GGAACTGATG A

21

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AATTCTAATA CGACTCACTA TAGGGAGAGG GTCGTCTGAC GAATGCCGA

49

35

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGTCTGTCAC ACAGTTGATG G

21

15

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AATTCTAATA CGACTCACTA TAGGGAGAGC TGAGTCTCTT TGATACCAA

49

30

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CGAAAATCCT GGAAC TGGCT G

21

10

(2) INFORMATION FOR SEQ ID NO: 7:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCGCGGCGTG GTCAACGT

18

25

(2) INFORMATION FOR SEQ ID NO: 8:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

ACGAGGAAGT TGAGATCG

18

5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTTGGTGGTC GATGGGCAG

19

20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GGTCGTCTGA CGAATGCCGA

20

35

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTGAGTCTCT TTGATACCAA

20

15

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACGCGGTATC ATCAAAGT

18

30

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTGAAGAAGT TGAAATCG

18

10

(2) INFORMATION FOR SEQ ID NO: 14:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GTCGGAAGTA GAACTGCG

18

25

(2) INFORMATION FOR SEQ ID NO: 15:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GACCCCGGCA CGCCGACT

18

5

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GACAAGCCGT TCCTGTGCC

19

20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GTCGGAAGTA GAACTGCG

18

35

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GGCACAGGAA CGGCTTGTC

19

15

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGCATCAGGA ACGGCTTGTC

20

30

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

AGTGGGTGTG CCCTACATCC TGGTAGCGCT G

31

10

(2) INFORMATION FOR SEQ ID NO: 21:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GGTGGGTGTA CCTTACATCC TGGTCGCACT T

31

25

(2) INFORMATION FOR SEQ ID NO: 22:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GGTCGGTGTG CCCTACATCC TGGTCGCGCT G

31

5

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GGTCGGCGTG CCGGCCCTGC TCGTGGCCCT G

31

20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GGTCGGCGTT CCGTACATCG TGGTCGCCCT G

31

35

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

GGTTGGCGTT CCTTACATCC TCGTTGCTCT T

31

15

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGTTGGCGTT CCTTACATCC TGGTTGCACT G

31

30

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

GGTAGGCGTT CCGTACATCA TCGTGTTCC G

31

10

(2) INFORMATION FOR SEQ ID NO: 28:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GGTAGGCGTT CCCTACATCG TCGTGTTCC G

31

25

(2) INFORMATION FOR SEQ ID NO: 29:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CTGCCAGGA ATTGACGAG

20

5

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

CCGCCAGGA GTTCGACGAG

20

20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

CCGCCAGGA GTTC

14

35

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TCTCAGTACG ACTTCCCGGG C

21

15 (2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TCTCAGTACG ATTTCCCGGG C

21

30 (2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

CCTCCAGGAG CTTCGACGTC

20

10 (2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CTCCGAGTAC GAGTTCCCGG GCGAC

25

25 (2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

CTGAGCAGGA CTACGAC

17

5

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

AACACCTACG ACTTCCCGGG C

21

20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GCTC

4

35

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

ATTG

4

15

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

TCTG

4

30

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

GCTG

4

10

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

TTGCTCTGTC GAGGAAGTGA TGA

23

25

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

TTGCCTCTGT CGAGGAACTG ATGA

24

5

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

TCGAGTCTGT CACACAGTTG ATGG

24

20

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

TCAAGTCCGT CGAGGACCTC ATGG

24

35

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

GCAACTCGGT CCTCGAGCTC ATGA

24

15

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

GCAAGCAGAT CCTTGAGCTC ATGC

24

30

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

TGGAGTCCGT CGAGCAG

17

10

(2) INFORMATION FOR SEQ ID NO: 49:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

AAGCGAAAAT CCTGGAAC TG GCTGGC

26

25

(2) INFORMATION FOR SEQ ID NO: 50:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

AAGCGAAAAT C

11

5

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

CATTACCGGC CGCGGA

16

20

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TATCACCGGT CGTGGC

16

35

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

TCGGCATTG CCCATCGACC ACCAAG

26

15

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

TCGGCATTG TCAGACGACC ACCAAG

26

30

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

TCGGCATCCG CCCGACCAGC ACCAAG

26

10

(2) INFORMATION FOR SEQ ID NO: 56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 56:

TCGGCATCCG CCCGTCCAGC ACCAAG

26

25

(2) INFORMATION FOR SEQ ID NO: 57:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 57:

TCGGCATCCG CCCGGAGACC ACCAAG

26

5

(2) INFORMATION FOR SEQ ID NO: 58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 58:

TTGGTATCAA AGAGACTCAG AAGT

24

20

(2) INFORMATION FOR SEQ ID NO: 59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 59:

TTGGTATCAA AGAGACCGCG AAAA

24

35

(2) INFORMATION FOR SEQ ID NO: 60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 60:

TCGGCATCAA GGCGACCACC AAGA

24

15

(2) INFORMATION FOR SEQ ID NO: 61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 61:

CATTAAGCCG CCCAGCACCA AGA

23

30

(2) INFORMATION FOR SEQ ID NO: 62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 62:

TCGGCATCCG TCCGACACCA CCAAGA

26

10

(2) INFORMATION FOR SEQ ID NO: 63:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 63:

TGGTTTGCTG CTGCGGGGCG

20

25

(2) INFORMATION FOR SEQ ID NO: 64:

30 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 64:

TGGTCTGTTG TTGCGTGGCA

20

5

(2) INFORMATION FOR SEQ ID NO: 65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 65:

CGGTCTGCTG CTGCGTGGTA

20

20

(2) INFORMATION FOR SEQ ID NO: 66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 66:

TGGTAACCTG CTGCGCTGGC A

21

35

(2) INFORMATION FOR SEQ ID NO: 67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 67:

CGGTCTGTTG CTCCGTGGCA

20

15

(2) INFORMATION FOR SEQ ID NO: 68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 68:

CGGGTCTGTT TGCTGCGTGG TG

22

30

(2) INFORMATION FOR SEQ ID NO: 69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: cDNA

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 69:

AGGTGTTCTG CTGCGCGGTA

20

10 (2) INFORMATION FOR SEQ ID NO: 70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: cDNA

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 70:

AGGTGTTCTG CTGCGTGGTA

20

25 (2) INFORMATION FOR SEQ ID NO: 71:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 71:

TTGCCTCTGT CGAGGAACTG ATGA

24

5

(2) INFORMATION FOR SEQ ID NO: 72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 72:

TCGAGTCTGT CACACAGTTG ATGG

24

20

(2) INFORMATION FOR SEQ ID NO: 73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 73:

TCAAGTCCGT CGAGGACCTC ATGG

24

35

(2) INFORMATION FOR SEQ ID NO: 74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 74:

GCAACTCGGT CCTCGAGCTC ATGA

24

15 (2) INFORMATION FOR SEQ ID NO: 75:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 75:

GCAAGCAGAT CCTTGAGCTC ATGC

24

30 (2) INFORMATION FOR SEQ ID NO: 76:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- 35 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 76:

TCGAGTCTGT CAAC

14

10

(2) INFORMATION FOR SEQ ID NO: 77:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

15

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 77:

TTGCTCTGTC GAGGAAGTGA T

21

25

(2) INFORMATION FOR SEQ ID NO: 78:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

30

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 78:

TGGAGTCCGT CGAGCAGCTG AT

22

5

(2) INFORMATION FOR SEQ ID NO: 79:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 79:

TGGAGTCCGT CGAGCAG

17

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(2) INFORMATION FOR SEQ ID NO: 80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 80:

TTGCTCTGTC GAGG

14

35

(2) INFORMATION FOR SEQ ID NO: 81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 81:

AAGCGAAAAT CCTGGAACTG GCTGGC

26

15

(2) INFORMATION FOR SEQ ID NO: 82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 82:

AAGCGAAAAT CCTGGAACTG

20

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(2) INFORMATION FOR SEQ ID NO: 83:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 base pairs
- 35 (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 83:

AAGCGAAAAT C

11

10

(2) INFORMATION FOR SEQ ID NO: 84:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 84:

TCGGCATTG TCAGACGACC ACCAAG

26

25

(2) INFORMATION FOR SEQ ID NO: 85:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 85:

TCGGCATTCTG CCCATCGACC ACCAAG 26

5

(2) INFORMATION FOR SEQ ID NO: 86:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 86:

TCGGCATTCTG TCAGACGACC ACCAAG 26

20

(2) INFORMATION FOR SEQ ID NO: 87:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 87:

TCGGCATCCG CCCGACCAGC ACCAAG 26

35

(2) INFORMATION FOR SEQ ID NO: 88:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 88:

TCGGCATTG CCCATCGACC ACCAAG

26

15 (2) INFORMATION FOR SEQ ID NO: 89:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 20 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 89:

TCGGCATCCG CCCGTCCAGC ACCAAG

26

30 (2) INFORMATION FOR SEQ ID NO: 90:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- 35 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 90:

TCGGCATCCG CCCGGAGACC ACCAAG

26

10

(2) INFORMATION FOR SEQ ID NO: 91:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs

15 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 91:

TCAGCATTAA GCCGCCAGC ACCAAG

26

25

(2) INFORMATION FOR SEQ ID NO: 92:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

30 (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 92:

TCGGCATCCG TCCGACACCA CCAAG

25

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(2) INFORMATION FOR SEQ ID NO: 93:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 10 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 93:

TTGGTATCAA AGAGACTCAG AAGT

24

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(2) INFORMATION FOR SEQ ID NO: 94:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 25 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 94:

TTGGTATCAA AGAGACCGCG AAAA

24

35

(2) INFORMATION FOR SEQ ID NO: 95:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- 5 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 95:

TCGGCATCAA GGCGACCACC AAGA

24